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Radioimmunoassay for Serum Thyroglobulin Designed for Early Detection of Metastases and Recurrencies in the Follow-Up of Patients with Differentiated Thyroid Carcinoma[1])

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Summary: A radioimmunoassay (RIA) for the measurement of thyroglobulin in human serum was developed and factors that influence sensitivity were investigated. In a comparison of 3 different labeling procedures (chloramine T, iodogen, lactoperoxidase) iodogen-prepared tracer proved to be slightly superior with respect to sensitivity and stability.

The shelf life of the tracer was improved by a protein-enriched buffer, which serves as a radical scavenger. The binding kinetics of tracer to antibody at different temperature ranges were examined, and the most rapid and complete binding was found at room temperature. For the preparation of standard curves, several artificial media were compared with thyroglobulin-free serum.

Second antibody separation was investigated and optimized. By employing sequential saturation, sensitivity of 0.75 µg/l (B_0–3 SD) and 50% intercept of less than 5 µg/l were achieved. The results of RIA measurements of thyroglobulin in 142 patients with papillary and follicular thyroid carcinoma after thyroidectomy and ^{131}I treatment were compared with ^{131}I whole-body scans. The results confirmed that serum thyroglobulin is an early indicator of recurrency.

Radioimmunoassay für Thyreoglobulin im Serum zur Früherkennung von Rezidiven und Metastasen in der Nachsorge von Patienten mit differenzierten Schilddrüsenkarzinomen

Zusammenfassung: Es wurde ein Radioimmunoassay (RIA) zur Messung von Human-Thyreoglobulin im Serum entwickelt und die Faktoren untersucht, die die Empfindlichkeit des Nachweises beeinflussen. Von den Markierungsmethoden mit Chloramin T, Iodogen und Lactoperoxidase zeigte der mit Iodogen gewonnene Tracer die höchste Empfindlichkeit und Stabilität.

Eine Untersuchung zur Aufbewahrung der Tracer zeigte, daß mit einer proteinreichen Lösung als Radikal-Fänger die Haltbarkeit der Tracer gesteigert wird. Die Kinetik der Bindung von Tracer an Antiserum bei verschiedenen Temperaturen ergab die schnellste Assoziation bei Raumtemperatur, sowie auch das höchste Ausmaß an Bindung in diesem Temperaturbereich. Verschiedene künstliche thyreoglobulinfreie Medien wurden untersucht und ihr Einsatz als Verdünnungsmedien für die Standardreihe geprüft.

Die Doppelantikörpertrennung wurde optimiert. Es resultierte eine Empfindlichkeit des Nachweises von 0,75 µg/l (B_0–3 SD) und ein 50% Intercept unter 5 µg/l.

Die Resultate des RIA für Thyreoglobulin bei 142 Nachsorge-Patienten mit papillärem und follikulärem Schilddrüsenkarzinom (Zustand nach Thyreoidektomie und ^{131}I-Behandlung) wurden mit denen der ^{131}I-Ganzkörperszintigraphie verglichen. Hierbei bestätigte sich die Bedeutung der Bestimmung von Thyreoglobulin als Frühindikator für das Vorliegen eines Rezidivs.

[1]) Supported by IOE (Tumorzentrum Heidelberg/Mannheim).

Introduction

Several methods have been published for the thyroglobulin assay (1–4). Radioimmunoassay has found wide application, and most assays employ the double antibody procedure.

For clinical use of the thyroglobulin assay, sensitivity and precision seem to be most important (5, 6). The assay is mainly employed in the follow-up of patients with differentiated thyroid carcinoma to detect early metastases and recurrencies.

^{131}I scans in connection with the measurement of serum thyroglobulin proved to be most reliable in the follow-up of those patients (7–11). Some authors (6, 12–14), have even proposed a reduction of scanning frequency after treatment if regular thyroglobulin measurements are performed. Since patients, after successful therapy of papillary and follicular thyroid carcinoma, should have no thyroglobulin in the serum (1, 5, 8, 9), the occurrence of metastases and recurrencies may be detected earlier by sensitive assays (5).

Therefore, our aim was to study the factors important for the sensitivity of thyroglobulin RIA. Furthermore we wanted to show the advantages of thyroglobulin RIA in the follow-up of thyroid carcinoma. Patients with elevated serum thyroglobulin levels but no clinical evidence (^{131}I scan, chest X-ray, palpation) of relapse were especially checked with a view to explaining the nature of the elevated thyroglobulin values.

Materials and Methods

Reagents

Human thyroglobulin was isolated from thyroid glands obtained at surgery and the purification was performed as previously described (4).

For the preparation of standards and for labeling, thyroglobulin was diluted in phosphate buffer, pH 7.4.

Other reagents (analytical grade, "pro analyse") were purchased from Merck AG, Darmstadt, FRG. ^{125}I was from Amersham Buchler GmbH, Braunschweig, FRG. Human serum albumin was from Behringwerke AG, Marburg/Lahn, FRG. All assays were performed in RIA-vials purchased from W. Sarstedt, Nümbrecht, FRG.

Preparation of antiserum

Antisera to thyroglobulin were prepared in rabbits injected at 4–8 week intervals over a total of 12 months. A suitable antiserum with a high titer was obtained in rabbit no. 1.

The antiserum was used at a dilution of 40,000-fold with phosphate buffer (0.06 mol/l, pH 7.4) containing human serum albumin (1 g/l), sodium azide (1 g/l) and EDTA (Titriplex III, 0.4 g/l) as diluent (working dilution). In addition, antiserum was purchased from UCB, Brüssel, Belgium.

Iodination of thyroglobulin

Chloramine T method

Thyroglobulin was iodinated according to a modification of the method by *Greenwood & Hunter* (15):

20 µg of purified thyroglobulin, 18.5 MBq of ^{125}I (IMS 30, Amersham Buchler) and 10 µl of a 1 g/l solution of chloramine T and 10 µl of phosphate buffer pH 7.4, 0.5 mol/l were mixed and reacted for 60 s. Then 50 µl of sodium metabisulfite (2.5 g/l) were added, immediately followed by 100 µl of dextran blue M_r 200000 (10 g/l) and the mixture was applied on a Sephadex G25 column (0.9 cm × 30 cm) and eluted with a Tris/HCl/albumin buffer, pH 7.4. Fractions (20 drops) were collected in a LKB 7000 fraction collector, and chromatography was complete after collecting 40 fractions. The first peak of radioactivity containing ^{125}I thyroglobulin was pooled.

For convenience, and without alterations in the results, this column was replaced by a ready to use column (Pharmacia PD 10, Pharmacia GmbH, Freiburg, FRG) and elution was performed with Tris-HCl-buffer, pH 7.4, containing 50 mg/l of human serum albumin. A fraction collector is not necessary.

Routinely, the tracer was diluted in the phosphate buffer used for diluting antiserum (1300 Bq/100 µl).

Lactoperoxidase method

The lactoproxidase method for iodination was employed according to the procedure given by BIO-RAD, Munich, FRG. The same amounts of thyroglobulin and iodine were used.

Iodogen method

Labeling by use of iodogen was performed according to *Wood* (16). Iodogen (2 µg, purchased from Pierce, Eurochemie BV, Rotterdam, NL) was dissolved in dichloromethane and placed in Eppendorf reaction vials. The dichloromethane was evaporated by N_2 and the tubes stored at −20 °C. Iodination was performed with the same amounts of thyroglobulin and ^{125}I as described above. Incubation was carried out at room temperature for 10 min with constant mixing on an automatic mixing device. All iodinations were done under a well ventilated hood.

Second antibody separation

Second antibody (RD 17, anti-rabbit IgG from donkey) was from Deutsche Wellcome, Burgwedel, FRG. This second antibody was diluted 1:24 in the buffer, used for diluting first antibody.

Radioimmunoassay

Before radioimmunoassay, all samples were screened for interfering anti-thyroglobulin autoantibodies (2, 9, 17, 18). Assay tubes and standards were arranged in duplicate and 200 µl of standards, controls and unknowns were added to the appropriate tubes, followed by 100 µl of thyroglobulin antiserum (1st antibody). The tubes were mixed, incubated as indicated (routinely 48 h), then 100 µl (1300 Bq) of ^{125}I labeled thyroglobulin was added to all tubes. Again the tubes were mixed and incubated for 48 h at room temperature. Then 100 µl of second antibody (donkey anti-rabbit IgG, RD 17, Deutsche Wellcome GmbH) was added and incubation continued for 2 h. The tubes were centrifuged for 10 min at 2000 *g*, at 4 °C to separate the bound and free fractions. The supernate was aspirated and discarded. The sediment was washed with 600 µl of washing solution (9 g/l NaCl). Again the tubes were centrifuged for 10 min at 2000 *g* and the supernates again aspirated and discarded. The radioactivity of the sediment was counted for 1 min or until 10000 counts were accumulated. The nonspecific counts (average of one duplicate in each assay) were subtracted from the average of each duplicate determination of sample and standard. The standard curve was constructed by plotting the ^{125}I labeled thyroglobulin versus the concentration of standards on 3-cycle semilogarithmic paper as shown in the figures.

Methodological results

Iodination

The use of different labelling procedures resulted in high incorporation of ^{125}I with the chloramine T and the iodogen method and only low incorporation using the lactoperoxidase method. With iodogen and chloramine T, 83% of ^{125}I was incorporated into thyroglobulin and 45% with lactoperoxidase. Specific activities of 370—740 kBq/µg were obtained.

Binding kinetics

The influence of time on the binding of ^{125}I labeled thyroglobulin tracer to the antiserum is illustrated in figure 1.

As shown in figure 1, iodogen-derived tracer resulted in better binding to the antiserum.

The binding of the tracer to the antibody is also influenced by temperature. Therefore, association kinetics were performed at 4—8 °C (refrigerator), room temperature, 37 °C and 42 °C (fig. 2).

As indicated in figure 2, incubation at room temperature resulted in the most rapid binding of tracer to the antibody. After 48 h, binding is nearly complete. Comparison of the binding kinetics of figure 2 with those of figure 1 shows that tracers prepared by the iodogen method display the same binding kinetics. From these experiments it was decided to perform all further experiments at room temperature.

Tab. 1. Influence of labelling method and storage on binding of ^{125}I labeled thyroglobulin.

Days after labeling	Total activity (Bq/100 µl tracer)	B_0 (Bq)	B_0/T	Non-specific binding Bq	(% of B_0)
Iodogen					
1	1301.5	733.8	0.56	35.7	4.9
7	–	576.6	–	28.5	4.9
14	1095.2	495.9	0.45	30.9	6.2
21	1018.6	419.8	0.41	31.5	7.5
28	909.7	329.6	0.36	27.8	8.4
	Δ = 391.8	Δ = 404.2	Δ = 0.20		Δ = 3.5
Chloramine T					
1	1360.5	613.3	0.45	34.2	5.6
7	1244.9	512.6	0.41	34.7	6.8
14	1126.0	461.5	0.41	35.2	7.6
28	1043.3	352.0	0.34	40.2	11.4
	Δ = 317.2	Δ = 261.3	Δ = 0.11		Δ = 5.8

B_0 = binding of thyroglobulin standard "0"
T = total activity
Δ = difference

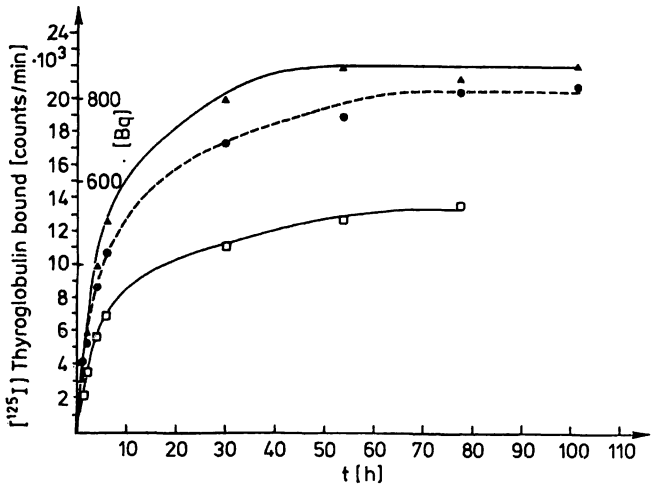

Fig. 1. Saturation kinetics at room temperature for ^{125}I thyroglobulin labeled according to the iodogen (▲ and ●) and lactoperoxidase (□) method.
In addition, binding of iodogen derived tracer to commercially available antiserum (– – –, UCB, Belgium) and to our antiserum (——) is shown.

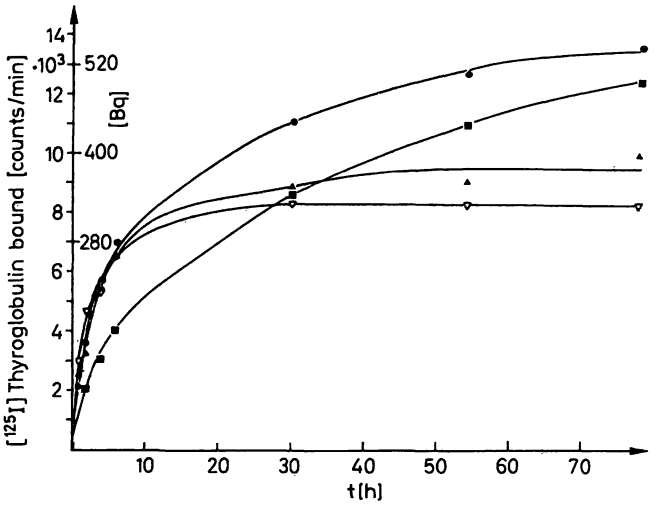

Fig. 2. Binding of ^{125}I thyroglobulin (labeled by lactoperoxidase) to the antibody at different temperature ranges:
■ = 4—8 °C, ● = room temperature, ▲ = 37 °C and ▽ = 42 °C.

Tracer stability

The labeling method influenced both the shelf life of the tracer and the unspecific binding. The influence of storage on the results is demonstrated in table 1.

The storage medium is of influence on the shelf life of the tracer. Protein is generally regarded as a radical scavenger. Storage of the tracer was therefore determined in routine buffer used for diluting antibodies and in a protein enriched buffer containing 10 g/l human serum albumin (tab. 2).

Tab. 2. Storage of ^{125}I thyroglobulin in phosphate buffer (0.06 mol/l, pH 7.4, containing 1 g/l human serum albumin, 1 g/l NaN_3 and 0.4 g/l EDTA) and in a protein-enriched buffer containing 10 g/l human serum albumin. These data were obtained 16 days after labeling.

Sample	Iodogen derived tracer with 10 g/l human serum albumin	Iodogen derived tracer with 1 g/l human serum albumin
NSB	24.3 Bq (5.3%)	36.0 Bq (7.7%)
B_0	456.5 Bq	470.2 Bq
IP-50%	5.0 µg/l	6.2 µg/l

NSB = non-specific binding
B_0 = binding of thyroglobulin standard "0"
IP-50% = 50% intercept point

Tab. 3. Binding of ^{125}I thyroglobulin to antiserum in different standard media.

Sample		Bq	Bq of NSB	% Sample	% NSB
Human thyroglobulin standard-0 in bovine serum		572.9	82.7	100.0	14.4
Protein/phosphate buffer with sodium-azide and EDTA-Na_2					
Human serum albumin	1 g/l	611.0	45.1	106.7	7.9
Human serum albumin	10 g/l	576.7	23.8	100.7	4.2
Human serum albumin	20 g/l	582.3	20.2	101.6	3.5
Human serum albumin	40 g/l	586.5	13.0	102.4	2.3
Patient C.+		581.4	10.0	101.5	1.7

NSB = non-specific binding
+ = patient C. is thyroidectomized with no signs of recurrence for more than two years after operation for medullary thyroid carcinoma

It is evident from table 2 that both the unspecific binding and the 50% intercept are lower in the protein enriched buffer. The use of different tracers resulted in different sensitivities (fig. 3).

Figure 3 indicates that the most sensitive tracer was obtained by the iodogen method, resulting in 50% intercepts from 3 µg/l – 5 µg/l.

Thyroglobulin standards

Since it is difficult to obtain sufficient amounts of thyroglobulin-free serum, several artificial media were checked for their suitability as thyroglobulin standard diluents (tab. 3).

As shown in table 3, a phosphate buffer (0.06 mol/l, pH 7.4) containing 10 g/l human serum albumin, 1 g/l NaN_3 and 0.4 g/l EDTA yielded a count rate very near to that observed for thyroidectomized patient "C", a patient with previous medullary thyroid carcinoma, followed by more than 2 years free from the disease. Buffer compositions containing higher concentrations of human serum albumin (20 g/l or 40 /l) may be used, but this becomes more expensive.

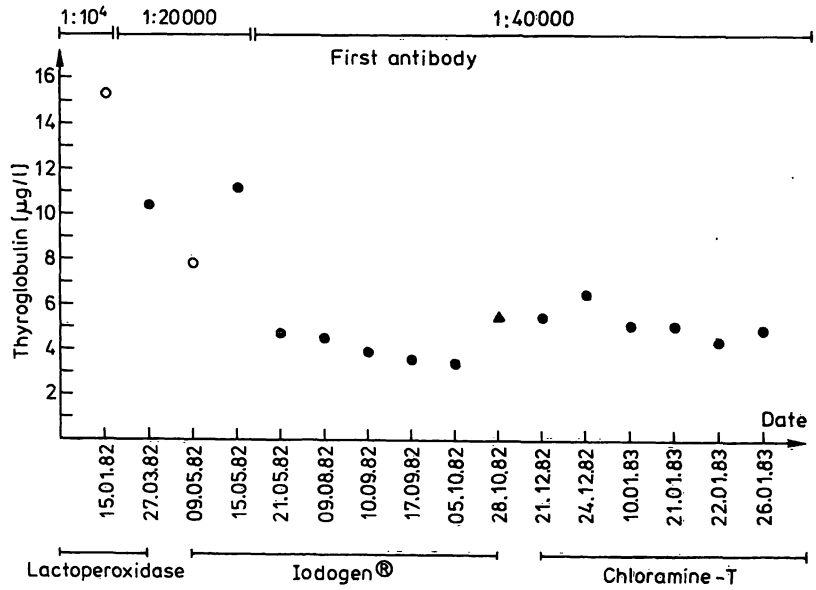

Fig. 3. Sensitivity of the assay determined by the 50% intercept point. Tracer was prepared by the lactoperoxidase, iodogen and chloramine T methods. Additionally the influence of antiserum dilution (1° antibody) on sensitivity, as well as different incubation modes (O = 3 + 3 days, ● = 2 + 2 days, ▲ = 1 + 1 day) are shown.

The medium for the standard influenced the shape of the standard curve. Thyroglobulin was therefore diluted in thyroglobulin-free serum and in the above mentioned buffer (10 g/l human serum albumin). The results are illustrated in figure 4.

As seen from figure 4, the standard curves are essentially superimposable.

Sensitivity and mode of incubation

Radioimmunoassays may be performed at equilibrium or with sequential saturation. For the purpose of detecting low concentrations of thyroglobulin in serum, different modes of incubation were compared (fig. 5).

It is apparent from figure 5 that sequential saturation results in increased sensitivity. 90% of the tracer is bound at 1 µg/l, about 80% at 2 µg/l and about 20% at 20 µg/l.

Separation of bound and free ligand

Separation of bound and free ^{125}I thyroglobulin (tracer) was performed by a double-antibody system (donkey anti-rabbit IgG, RD 17, from Deutsche Wellcome GmbH).

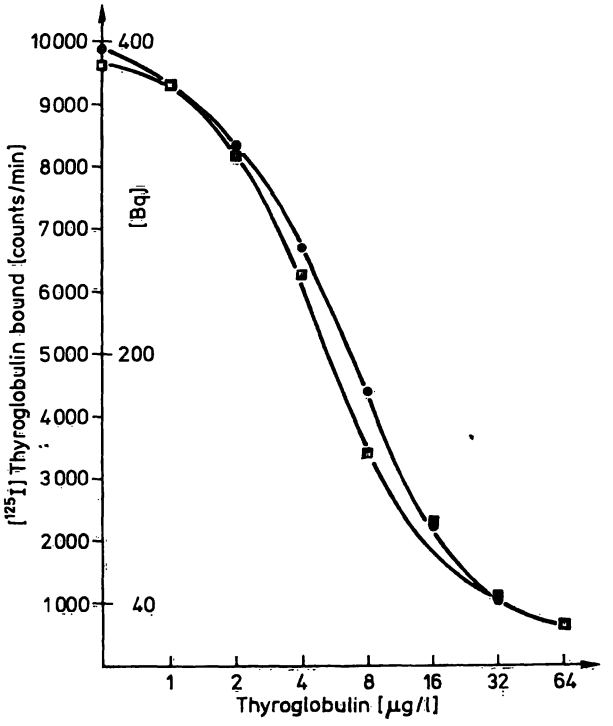

Fig. 4. Dilution of standards in thyroglobulin-free serum (●, pool from totally thyroidectomized patients) and in phosphate buffer with protein, 10 g/l, sodium-azide and EDTA (■).

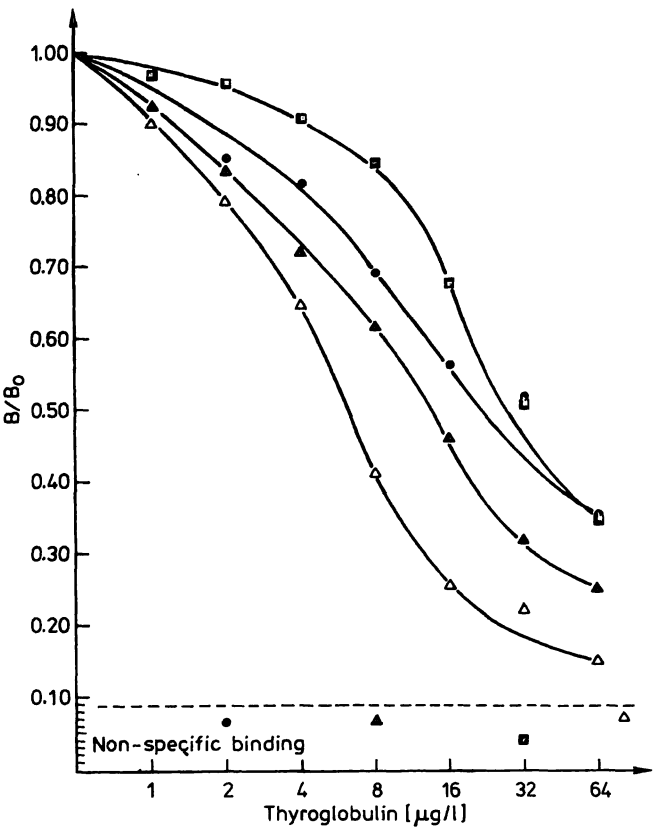

Fig. 5. Displacement of ^{125}I thyroglobulin (y-axis) by unlabeled thyroglobulin (x-axis). Incubation was performed at equilibrium with 48 h (○) and 72 h (▲) at room temperature and with 96 h (□) at 4–8 °C. Additionally sequential saturation is demonstrated with 2 days incubation of standards with antiserum and another 2 days with ^{125}I-thyroglobulin added (△).
Second antibody separation in all assays was performed at room temperature. Values of nonspecific binding (NSB) are shown below the dotted line.

It was necessary to first optimize the amount of normal rabbit serum included in the antiserum dilution.

The second antibody (anti-rabbit IgG) was diluted 1:24. A precipitation experiment with ^{125}I thyroglobulin and increasing amounts of first antibody containing normal rabbit serum (4 or 6 ml/l) was performed (fig. 6).

The addition of normal rabbit serum (6 ml/l) to the first antibody resulted in extremely high coefficients of variation (fig. 6), when 100 µl first antibody and 100 µl second antibody were used.

On the other hand, when 4 ml/l normal rabbit serum were added to the first antibody, and 100 µl first antibody and 100 µl second antibody were employed, a plateau of precipitated counts could be observed. Therefore, for further experiments 4 ml normal rabbit serum per liter first antibody solution were used.

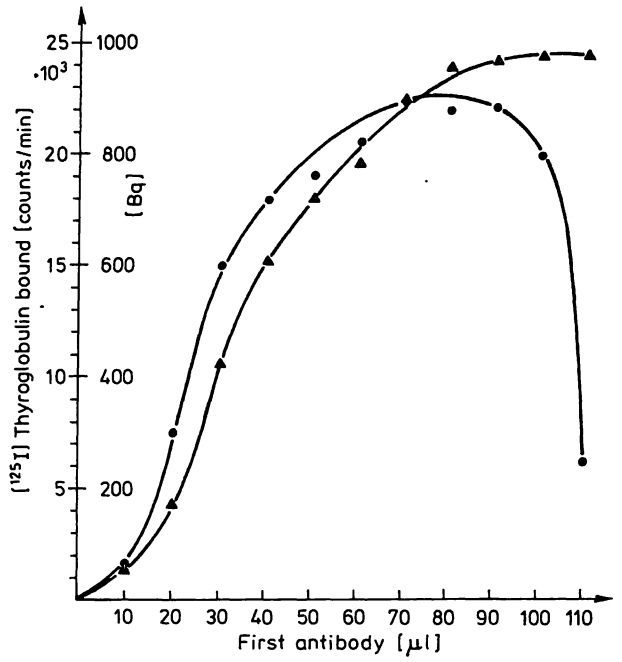

Fig. 6. Precipitation experiment with ^{125}I thyroglobulin and different amounts of first antibody containing normal rabbit serum (4 ml/l, ▲ or 6 ml/l, ●).
The tube contained 200 µl thyroglobulin-free medium, 100 µl ^{125}I thyroglobulin (1300 Bq), 10–110 µl first antibody, and was incubated for 48 h at room temperature. Then 100 µl second antibody from donkey (1:24, purchased from Deutsche Wellcome GmbH) was added and incubation continued for another 2 h. All tubes were filled to 510 µl with standard phosphate buffer. (0.06 mol/l, pH 7,4, containing 1 g/l human serum albumin, 1 g/l NaN_3 and 0.4 g/l EDTA).

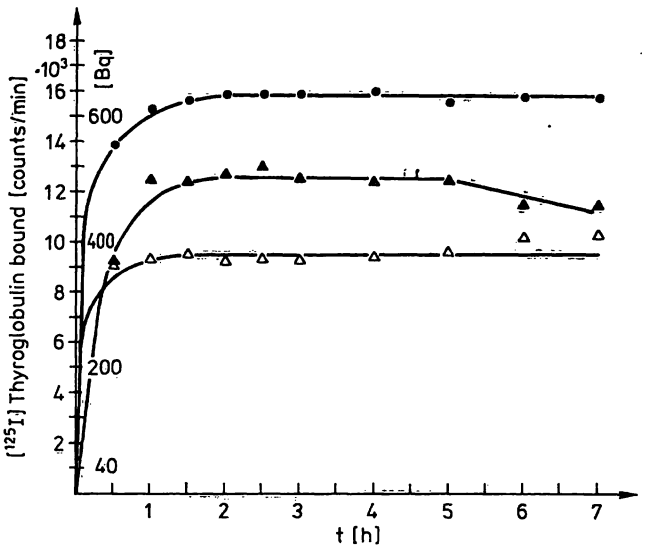

Fig. 7. Precipitation of ^{125}I thyroglobulin by second antibody from donkey (1:24, purchased from Deutsche Wellcome GmbH) at different temperature ranges: ▲ = 4–8 °C (refigerator), ● = room temperature and △ = 42 °C.

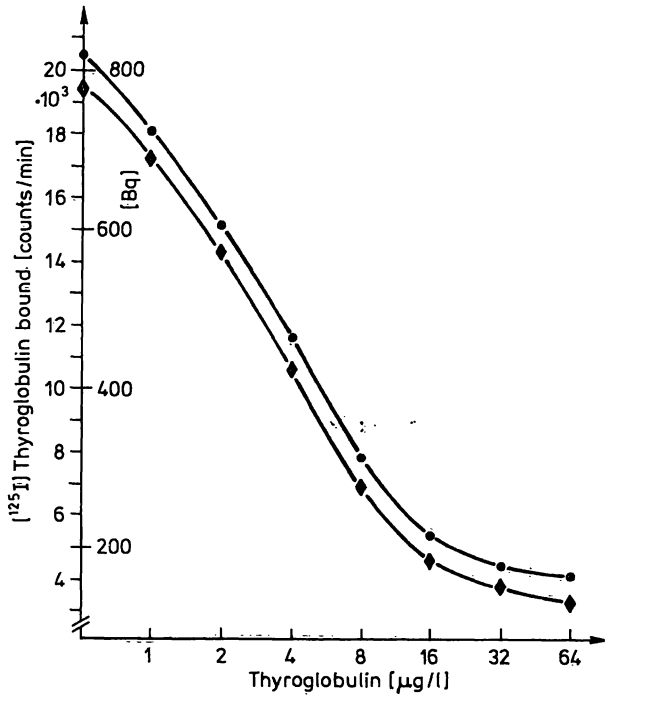

Fig. 8. Effect of washing (▲) and not-washing (●) the second antibody precipitate on the shape of standard curve. In each case nonspecific counts were subtracted.

As second antibody precipitation might be influenced by the incubation temperature, different temperature ranges and incubation periods were investigated (fig. 7).

It is evident from figure 7, that 2 h of incubation at room temperature was optimal.

Washing

After second antibody separation, some laboratories wash the precipitate. The effect of washing on the second antibody separation was investigated (fig. 8).

As can be seen from figure 8, washing with 600 µl of 9 g/l NaCl solution reduces nonspecific binding and alters the shape of standard curve at higher concentrations of unlabeled thyroglobulin. Therefore, washing was performed in further experiments because high nonspecific binding increases the imprecision of the results.

Quality control

Parallelism

A serial dilution was made of 2 patient sera containing thyroglobulin of about 30 µg/l. Thyroglobulin-free serum was used as diluent. The 2 curves plotted as a function of the dilution display parallelism to the standard curve (fig. 9).

However, some discrepancies exist in the high concentration range above 32 µg/l.

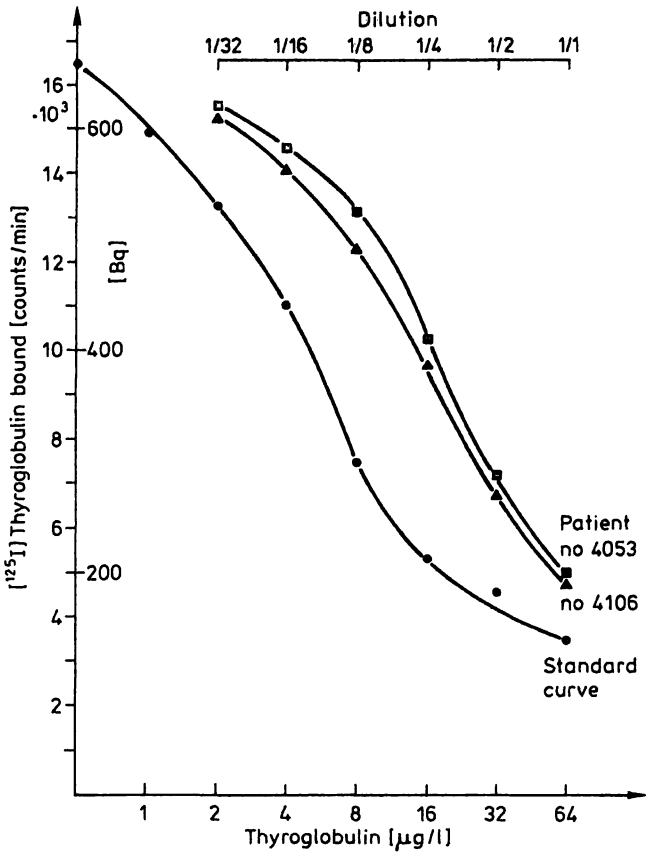

Fig. 9. Displacement of ^{125}I thyroglobulin from the antiserum by thyroglobulin-standards (●) and by dilution of patient sera (□ and ▲). Patient sera were diluted in thyroglobulin-free human serum. Standards were diluted in phosphate buffer containing serum albumin, 10 g/l, sodium azide and EDTA.

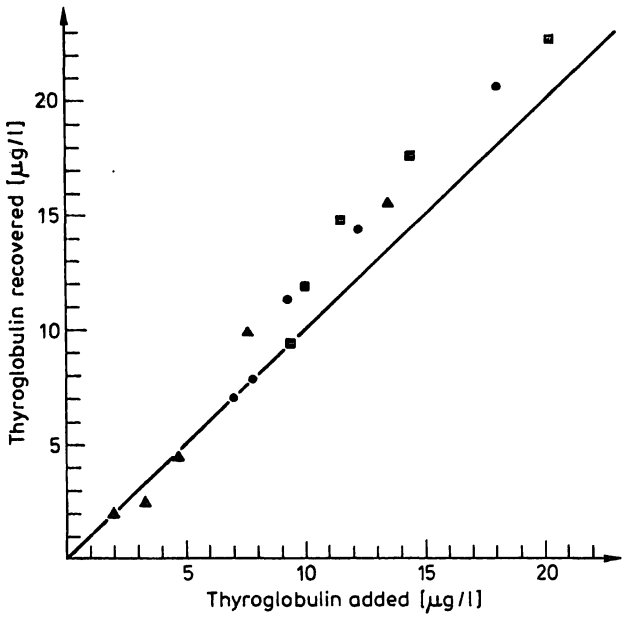

Fig. 10. Thyroglobulin was added to 3 different normal human sera (▲, ● and □). The amount of thyroglobulin added is shown on the x-axis, and the thyroglobulin recovered on the y-axis.

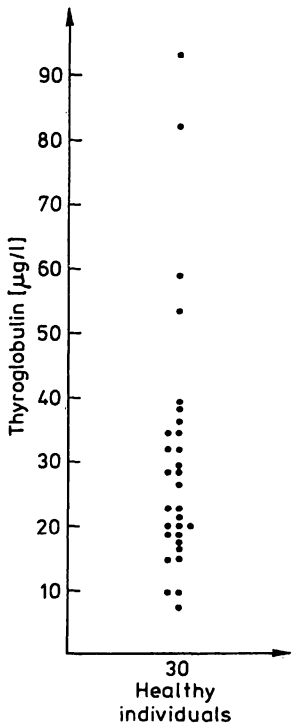

Fig. 11. Thyroglobulin serum concentration in 30 healthy individuals aged 20—40 years.

Recovery

An increasing amount of thyroglobulin was added to three serum samples with different thyroglobulin concentrations (fig. 10).

Figure 10 reveals that recovery is slightly higher than 100%.

Imprecision

Radioimmunoassays are known for their different precision at different concentrations of unlabeled ligand. For that reason, a precision profile was determined. The RIA was performed as usual (2d + 2d + 2h at room temperature). For each standard concentration, 12 measurements were performed. A typical U-shaped precision profile was obtained with coefficients of variation between 9% (1 µg/l), 3% (4 µg/l) and 5% (64 µg/l). The standard deviation is less than 0.2 µg/l.

Reference

30 healthy individuals 20—40 years of age exhibited serum thyroglobulin levels demonstrated in figure 11.

Sensitivity

The sensitivity of the thyroglobulin RIA was evaluated using the usual statistical test: 11 measurements of the radioactivity of the bound fraction were obtained for the standard without any detectable thyroglobulin. The mean, the standard deviation and the coefficient of variation were then calculated. The

minimum detectable concentration of thyroglobulin was then read off the standard curve as mean counts at zero concentration minus 3 standard deviations. This gave a sensitivity of 0.75 µg/l. RIA for this experiment was performed as usual: 2d + 2d + 2h at room temperature.

Interassay coefficient of variation

A control serum containing about 2.5 µg/l of thyroglobulin was determined in 8 consecutively performed assays. An interassay variation coefficient of 7.8% was obtained.

Clinical results

Patients

140 patients with papillary or follicular thyroid carcinoma and 2 patients with anaplastic thyroid carcinoma were selected in order to compare thyroglobulin radioimmunoassay with ^{131}I total body scans. Patients were only included if at least 1 scan and simultaneous serum sample without interfering antithyroglobulin autoantibodies were available.

All patients had undergone thyroidectomy as well as ^{131}I therapy and showed no residual thyroid tissue. There were 107 females and 35 males and mean age was 49.6 ± 13.8 years.

In 109 patients, clinical and radiological (chest X-ray, ^{131}I total body scan) investigation presented no signs of local or distant recurrence. 7 patients showed local relapse and 26 distant metastases.

Thyroglobulin radioimmunoassay and ^{131}I total body scan (tab. 4)

As indicated in table 4, thyroglobulin RIA was superior to ^{131}I total body scan in 3 of 7 cases with local recurrence and in 9 of 26 patients with distant metastases. In contrast ^{131}I total body scan was superior to thyroglobulin RIA in 2 of 26 patients with distant metastases.

Of 109 patients without evidence of relapse by clinical and radiological (chest X-ray, and ^{131}I total body scan) investigation, 17 showed elevated thyroglobulin serum levels (>6.4 µg/l).

Discussion

For the labeling of thyroglobulin, most authors use the chloramine T method (1–3, 19, 20). In addition, the lactoperoxidase method (17, 19, 21, 22) and the iodogen technique (23, 24) are employed. According to our data, the iodogen labeling procedure was slightly superior to chloramine T and lactoperoxidase; satisfactory ^{125}I incorporation, high specific activity and low nonspecific binding were obtained. The lactoperoxidase technique was relatively inefficient. The low incorporation of ^{125}I into thyroglobulin by this method may be explained by the fact that some of the radioactive iodine was incorporated into the enzyme.

In contrast to our findings, *Schlumberger & van Herle* (19) reported that lactoperoxidase-labeled tracers yielded higher binding and more prolonged stability than chloramine T tracers. The storage of the tracer was improved by using a protein-enriched buffer serving as tracer diluent (10 g human serum albumin per l tracer solution) and radical scavenger.

Schlumberger & van Herle (19) and others (21, 25) stipulated that ^{125}I labeled thyroglobulin should be rechromatographed weekly on a Sephadex G200 column. Our data, however, suggest that weekly rechromatography is not necessary. Iodogen derived tracer for example may be used for more than 4 weeks with no need for rechromatography. This disagreement with our data may be explained by the very high ratio of ^{125}I to thyroglobulin used by

Tab. 4. Thyroglobulin radioimmunoassay and ^{131}I total body scan findings in the follow-up of 142 patients with thyroid carcinoma.

Disease state in the follow-up of 142 patients with thyroid carcinoma	HTg-RIA ⊕ ^{131}I scan ⊕	HTg-RIA ⊕ ^{131}I scan ⊖	HTg-RIA ⊖ ^{131}I scan ⊕	HTg-RIA ⊖ ^{131}I scan ⊖
No signs of local recurrence or distant metastases	–	17	–	92
Local recurrence	4	3	–	–
Distant metastases	13	9	2	2

HTg-RIA ⊕ = serum thyroglobulin above the critical level of 6.4 µg/l
HTg-RIA ⊖ = serum thyroglobulin below the critical level of 6.4 µg/l
^{131}I scan ⊕ = positive ^{131}I total body scan in the follow-up
^{131}I scan ⊖ = negative ^{131}I total body scan in the follow-up

Schlumberger & van Herle (19): 74 MBq ^{125}I and only 5 µg thyroglobulin were used together with heavy oxidation of the protein (25 µg chloramine T oxidizing for 90 s).

Our iodinations were all performed with a ratio of ^{125}I to thyroglobulin which is 16-fold lower (18.5 MBq ^{125}I and 20 µg thyroglobulin). Using the chloramine T method, we only employ 10 µg chloramine T and oxidation was stopped after 60 s. For preparation of the standard curve, bovine serum should not be used as standard diluent, because it gives rise to high non-specific binding (14%).

Human serum devoid of thyroglobulin is the best standard diluent, though it is difficult to obtain a sufficiently large pool. Therefore, different buffers were tested as substitute. A phosphate buffer with protein (human serum albumin, 10 g/l), sodium azide and EDTA yielded very similar binding and non-specific binding results compared with thyroglobulin-free serum (fig. 5). *Bodlaender* et al. (2) also employ phosphate-buffered saline solution (73 g/l bovine serum albumin) for preparation of standard curve.

Sequential saturation for the radioimmunoassay of thyroglobulin is mostly used (2, 3, 21, 26). However, the reported incubation periods are much shorter (2, 3, 26, 27).

An attempt was also made to determine the optimal incubation temperature. From several experiments (fig. 2, 5, 7) it was decided to perform all incubation steps at room temperature. Some authors, however, incubate (first and second incubation) at 4 °C (3, 21), others at 37 °C and 25 °C, respectively (2, 27), or 37 °C and room temperature, respectively (26). As diagrams of binding kinetics have not been published by these authors, improved sensitivity may be obtained by prolonging the first and second incubation period and by determination of the correct incubation temperature. In our assay, we use 2 days + 2 days at room temperature. It is evident from this evaluation that every double antibody system consisting of antiserum, carrier γ-globulin and second antibody has to be checked carefully as far as dilution of antiserum, concentration of carrier γ-globulin and incubation temperature are concerned. Satisfactory results were obtained by adding 4 ml normal rabbit serum to 1 l of the first antibody solution (antiserum 1:40000). The necessary incubation time for second antibody from donkey (purchased by Deutsche Wellcome GmbH, diluted 1:24) was only 2 h at room temperature. In contrast to our findings, *Benita* et al. (3) reported that by use of second antibody from donkey an incubation period of 16–20 h is required (incubation temperature not mentioned).

There was parallelism between dilutions of the standards and of patient sera using thyroglobulin-free serum as diluent. The recovery of added thyroglobulin was nearly 100% at concentrations ranging from 2 µg/l to 10 µg/l and slightly above 100% at higher concentrations.

The accuracy in the high concentration range (16 µg/l – 64 µg/l) depended on washing the precipitate after second antibody separation. According to *van Herle* et al. (1) washing the precipitate is not necessary. This discrepancy may be due to the different assay volumes used.

The precision profile of our assay is typically U-shaped, as is usual for radioimmunoassays.

Intraassay coefficient of variation ranged from 3% to 9%. The interassay coefficient of variation for a patient sample (thyroglobulin: 2.5 µg/l) was 7.8%.

With the usual mode of incubation (2d + 2d + 2h at room temperature) a sensitivity of the assay of 0.75 µg/l was achieved, and the 50% intercept ranged from 4 µg/l to 7 µg/l (antiserum 1:40000, labeling method: iodogen or chloramine T, incubation periods as usual). These findings contrast with the data previously published (tab. 5).

Tab. 5. Sensitivity of published thyroglobulin radioimmunoassays as determined by 50% intercept point.

Author	Year	50% intercept-point of thyroglobulin RIA (µg/l)	References
Van Herle et al.	1973	30.0	(1)
Van Herle & Uller	1975	27.0	(29)
Bodlaender et al.	1978	45.0	(2)
Gembicki et al.	1981	65.0	(28)
Benita et al.	1981	100.0	(3)
Schlumberger & Van Herle	1982	25.0	(19)

The reference range is in accordance with the literature (8, 30). However, lower normal human thyroglobulin ranges have been reported (13, 17, 21).

For discrimination of patients in remission and those presenting local or distant metastases, a critical level (cut-off) of 6.4 µg/l was determined by statistical means (31).

Definite evidence of local recurrence in our 7 patients was always associated with elevated thyroglobulin serum levels (>6.4 µg/l). In 3 of these 7 instances the ^{131}I total body scans, however, were negative.

In 26 subjects with distant metastases, high serum thyroglobulin levels and positive ^{131}I scans were found in 13 (50%) cases. In 9 patients (34%) with elevated serum thyroglobulin the ^{131}I scans were negative. In 2 examinations (8%), positive ^{131}I scans were associated with thyroglobulin levels lower than 6.4 µg/l. An additional 2 subjects (8%) showed both negative ^{131}I scans and low serum thyroglobulin values (<6.4 µg/l).

Thus in 12 of 14 cases (86%) with negative ^{131}I scintigrams and definite recurrent thyroid carcinoma, the diagnosis was verified by the measurement of thyroglobulin.

In 92 of 109 patients (84%) presenting no clinical evidence of relapse, both serum thyroglobulin and ^{131}I scans were negative. However, in 17 of these 109 subjects elevated serum thyroglobulin levels were found. In contrast to other tumour marker tests we do not regard these elevated thyroglobulin levels as "false positive". According to our experience these patients represent a "high risk group" since 2 of these 17 subjects have since revealed recurrent thyroid carcinoma.

These findings suggest that a sensitive thyroglobulin radioimmunoassay is able to predict a relapse of thyroid carcinoma at a time when all other clinical data are still inconspicuous. Summarizing all the results, we can postulate:

1. Serum thyroglobulin is a very reliable and early indicator of recurrency.
2. Sensitive thyroglobulin radioimmunoassay helps to reduce the number of ^{131}I control scans, especially since *Hüfner* et al. (32) have demonstrated that routine ^{131}I scan is of very low efficiency.
3. With the help of thyroglobulin assay we can pick out "high risk patients". Those patients presenting elevated serum thyroglobulin values (after thyroidectomy and ^{131}I therapy) but no further clinical evidence of relapse should be subjected to close and detailed screening.
4. However, in those cases where thyroglobulin autoantibodies are present, our thyroglobulin radioimmunoassay is not usable.

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